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EPIGENETIC SHAPING OF HIPPOCAMPAL VULNERABILITY IN PERINATAL ASPHYXIA IN RAT

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ABSTRACT

Hypoxic-ischemic encephalopathy secondary to asphyxia at birth is a leading cause of neonatal morbidity with early- and long-term consequences, especially on more vulnerable areas of the brain, such as hippocampus. Asphyxia severity can be influenced by concurrent maternal factors, like dietary factors, that could either mitigate or aggravate it. Trans-resveratrol (tRESV), a polyphenol with powerful antioxidant properties present in food and accessible as a maternal dietary supplement, is a potential neuroprotective candidate in case of excitotoxic mechanisms triggered by asphyxia in the neonate brain. Our group investigated the epigenetic mechanisms of brain injury and inflammation involved in asphyxia in immature brain and examined the effects of tRESV when present as a dietary habit in maternal daily intake. We used an experimental asphyxia model consisting in a 90-min exposure to hypoxia and hypercapnia in postnatal day 6 Wistar rats taken from mothers with/without tRESV supplement. The levels of hippocampal S-100B protein, interleukin 1 beta (IL-1b) and tumour necrosis factor alpha (TNFα) were measured at 24-48 hours. Also, we determined the expression of selected microRNAs (miR15a, miR124, miR132, miR134, and miR146) 24 h post-asphyxia, to investigate the epigenetic hippocampal response triggered by asphyxia and tRESV supplement. We report here an epigenetic mechanism involved in the hippocampal response to asphyxia and a neuroprotective effect of tRESV, sustained by a reduction in post-asphyxia neural inflammation and injury, with an increased tolerance to asphyxia and a potential influence on neuronal maturation. Our results encourage the use of tRESV in the maternal diet for its protective potential on the offspring's post-asphyxia outcome and prompt to further exploration of maternal diet influence on neonatal brain pathology, with subsequent impact on the quality of life.

Keywords: perinatal asphyxia, hippocampus, neural injury, epigenetics, maternal diet, trans-resveratrol

INTRODUCTION

The influence of environmental factors like diet or other metabolic conditions are now recognized as epigenetic modulators of genes function. One clinically impactful example is the influence which maternal diet can have on neonatal neurodevelopment and neuroplasticity [LaRosa, 2017]. Recent research studies investigate the way the maternal diet modulates the severity of perinatal brain pathologies like hypoxic-ischemic encephalopathy consecutive to asphyxia at birth [Sanches, 2012; Black, 2015; Loren, 2005].

PERINATAL ASPHYXIA

Perinatal asphyxia (PA) is a condition characterized by an impairment of exchange of the respiratory gases (oxygen and carbon dioxide) resulting in hypoxemia and hypercapnia, accompanied by metabolic acidosis [Bax, 2007] in the fetus or neonate. The severity of asphyxia is defined as a series of cardio-respiratory changes, including hypoxia (decreased pO2), hypercapnia (increased pCO2), metabolic acidosis (increased lactate), impaired blood gas exchange and ischemia, with consecutive decreased oxygen delivery to the tissues. Perinatal asphyxia is reported to occur in 1 to 6 per 1,000 live full-term births [de Haan M, 2006] but it may be more common in preterm babies and is considered the third main cause of neonatal death after preterm delivery and perinatal infections [Lawn, 2005]. Asphyxia injury can involve virtually every organ or system of the body however, the brain is the most vulnerable structure to its effect, exhibiting the most serious short-term and long-term sequelae. Perinatal asphyxia involving the brain can lead to hypoxic-ischemic encephalopathy (HIE), characterized by clinical and laboratory evidence of acute or subacute brain injury due to asphyxia. Clinical and laboratory findings in PA include metabolic acidosis (pH < 7.0 and base deficit ≥ 12 mmol/L) in umbilical artery blood sample at birth, moderate or severe signs of encephalopathy, cerebral palsy of spastic quadriplegia or dyskinetic type later in development [Task Force, ACOG, 2017]. Virtually every region of the brain is susceptible to the effects of PA but some brain regions are more vulnerable to this injury than others. In premature neonates who are also of a low birth weight the highest risk is of periventricular leukomalacia and germinal matrix haemorrhage. Term neonates who undergo birth asphyxia or hypoxia-ischemia late in gestation tend to have injury in the deep grey matter, hippocampus, brainstem, and thalamic regions (Swarte et al., 2009).

The response of the developing brain to hypoxia-ischemia is a multi-step process occurring in a time-dependent fashion. Within the first few hours there is a regionally-specific increase of cerebral blood flow, followed by its decrease. Subsequently, energy depletion causes excitotoxicity and generation of free radicals resulting in increased apoptotic and necrotic cell death and oedema formation. A secondary phase of injury occurs in the following hours and days, accompanied by a neuroinflammatory response, mitochondrial permeabilization, and a loss of cerebral autoregulation which can also lead to increased free radical production [Baburamani, 2012]. The full extent of these lesions becomes apparent over the course of days to years after the initial insult (Fig. 1).

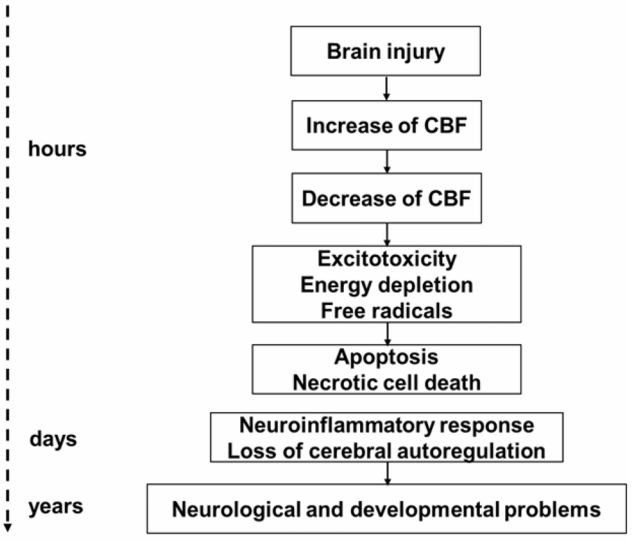

FIGURE 1. *Asphyxia's effect on the brain in the term neonate; CBF – cerebral blood flow.*

Methods to assess the impact of PA on neonatal brain have been developed and they are useful in predicting short and long-term outcomes and in selecting adequate candidates for specific therapies. In the clinical setting, neonatal examination and clinical course, monitoring general movements, early electrophysiology testing, cranial ultrasound imaging, Doppler blood flow velocity measurements, magnetic resonance imaging (MRI) and MR microscopy are widely used. The neonatal brain MRI provides detailed information about lesion patterns in HIE allowing for earlier and more accurate prediction of long-term outcome [Martinez, 2011]. Serum biomarkers such as lactate dehydrogenase [Karllson, 2010] in blood of the new-born infant during the first 12 h after birth have both diagnostic and predictive utility. Potential biomarkers of brain injury that have been described to assess the extent of PA [Mir, 2014] are presented in Table 1. One of the best studied biomarkers in the diagnosis and prediction of brain injury related to PA is protein S100 beta [Thorngren-Jerneck K, 2004], a calcium binding protein which is a major component of the cytosol in various cell types but is particularly found in high concentration in glial cells. After a hypoxic-ischemic insult of the brain

the levels of this protein sharply increase locally within the brain, in the cerebro-spinal fluid (CSF), in the blood and urine of the neonate. S100 beta immunoassay kits are commercially available and can detect this protein in urine, blood, CSF, amniotic fluid, and saliva. A recent study showed that S100 beta concentration cut-off of 0.41 mcg/L has a sensitivity of 91.3% and a specificity of 94.6% for predicting the development of HIE. The sensitivity and specificity increased to 100 and 98.8%, respectively, when studied samples were collected at 4–72 h after birth [Gazzolo D, 2009].

The principle to postnatal therapeutic interventions in HIE is therapeutic hypothermia. Controlled hypothermia prevents progression of injury after the initial asphyxia insult. Therapeutic hypothermia improves outcomes of death and disability and should be started as soon as possible, ideally within 3 hours of birth to achieve better neurodevelopmental outcomes.

TABLE 1. *Potential biomarkers to assess post-asphyxia brain injury in the neonate*

Brain-specific biomarkers
Glial fibrillary acidic protein (GFAP) **Ubiquitin carboxyl-terminal esterase L1 (UCH-L1)** **Neuron specific enolase (NSE)** **S-100 beta**
General biomarkers
Tumour Necrosis Factor- α (TNF-α) **Interleukin-6 (IL-6)** **Interleukin-1b (IL-1b)**

In our study we used a perinatal asphyxia model in postnatal day 6 Wistar rats from mothers who received a standard diet and a trans-resveratrol (tRESV)-supplemented diet. Exposure to hypoxia (9% O2) and hypercapnia (20% CO2) for 90-min, at 37°C, was induced in accordance with Helmy [Helmy, 2011]. A correspondent exposure to room air was used for the control normoxia group. Experimental setup is showed in Fig. 2 from Isac et al [Isac, 2017]. From hippocampi harvested at 24-48 hours post-exposure, we determined by ELISA neuroinflammation and neural injury markers: tumour necrosis factor alpha (TNFα), interleukin 1 beta (IL-1b) and S-100B protein (S-100B). All these makers were significantly increased by asphyxia and this effect was reversed by tRESV, as shown in Fig. 3 [Isac 2017]. Also, by reverse transcription PCR (RT-PCR), we investigated the expression of microRNAs miR124, miR132, miR134, miR15a and miR146, selected for their involvement in the epigenetic control of apoptosis, tolerance to asphyxia, angiogenesis and neuronal maturation and inflammation, as showed in the following section. All detailed experimental procedures were described in Isac et al. [Isac, 2017].

EPIGENETIC IMPACT ON BRAIN TOLERANCE TO PERINATAL ASPHYXIA

Environmental factors and diet are well-known for their epigenomic effects, especially early during development, but these effects are extended during the whole life, with trans-generational impact.

Trans-resveratrol is a food polyphenol accessible in the normal diet, as it is found in grape skin, blueberries, pistachios and peanuts [Liu, 2016]. Recently, trans-resveratrol was promoted for its antioxidant properties with protective potential in brain disease models, like Parkinson's disease [Chen, 2007], seizures [Hoda, 2016], or neuroinflammation associated with high-fat intake [Bernier, 2016]. As there were no previous studies to show the effects of tRESV maternal intake on offspring's post-asphyxia brain lesion, we considered it an important issue worth to be investigated [Isac, 217]. We chose to use in our study low to moderate dose trans-resveratrol, administered over a long period of time, in order to study its dietary preventative qualities rather than its therapeutic use in response to acute injuries, as PA.

The changes at epigenome level, associated with different brain pathologies [Yu, 2015], modulate

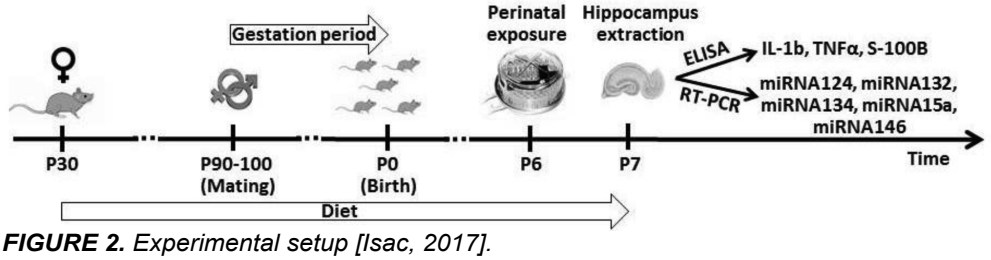

FIGURE 2. *Experimental setup [Isac, 2017].*

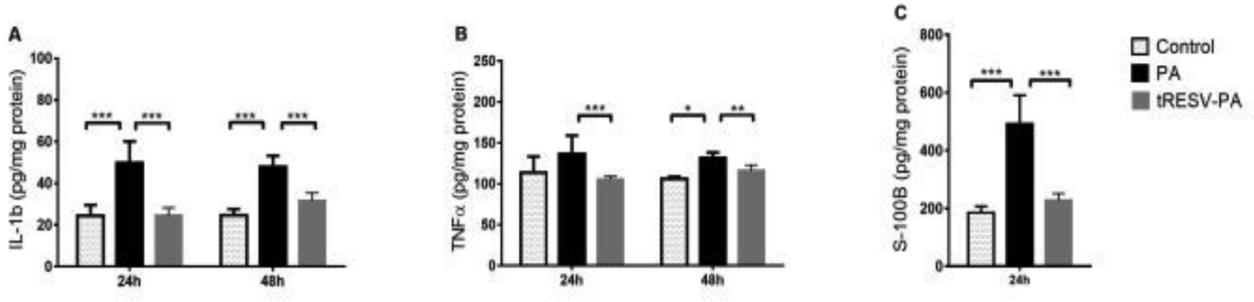

FIGURE 3. *Post-asphyxia levels of IL-1b (A), TNFα (B) and S-100B (C) in control, asphyxia (PA) and asphyxia tRESV-treated groups. Bars represents means ± SEM. * for p = 0.05-0.01, ** for p = 0.01-0.001 and *** for p< 0.001 [Isac, 2017].*

the genes expression by histone acetylation, DNA methylation or by small non-coding RNA, like microRNAs (miRNA) [Bartel, 2004]. Identifying epigenetic biomarkers for specific pathologies could enable early diagnosis, non-invasive monitoring and also more efficient preventive and therapeutic strategies, important in high-incidence conditions like neonatal asphyxia and its secondary hypoxic-ischemic encephalopathy. In our study we aimed to identify specific miRNAs that could be used as epigenetic markers of brain response to asphyxia and the way they are influenced by a concurrent exposure to maternal dietary factors, like tRESV. Thus, we determined miR124, miR132, miR134, miR15a and miR146 levels, known to be associated with brain disorders [Yu, 2015; Jimenez-Mateos, 2011; Gao, 2010; Gao, 2015; Iyer, 2012]. The most representative brain miRNA is miR124, known for its involvement in homeostasis of synaptic plasticity, axogenesis and neuroinflammation [Hou, 2015]. In our study we have shown that miR124 in the hippocampus is down-regulated by an acute stress such as PA, with impact on the homeostatic synaptic plasticity, with no significant impact from tRESV maternal diet [Isac, 2017] (Fig. 4).

Down-regulation of miR132 is associated with raised neuronal tolerance to hypoxia/ischemia [Jimenez-Mateos, 2011]. We found that immature hippocampus exposed to PA could adapt to potential future hypoxia episodes, by increasing its tolerance to ischemia, secondary to down-regulation of miR132 [Isac, 2017]. We also showed that tRESV further down-regulate miR132 when compared to pups exposed to asphyxia from mothers who received normal diets, thus tRESV increasing hippocampal tolerance to asphyxia.

It is known that miR134 has an impact on memory formation and ischemic injury [Gao, 2010; Chi, 2014]. The down-regulation of hippocampal miR134 post-asphyxia revealed in our study, associates with the impairment in memory formation secondary to alteration in synaptic plasticity found in clinical

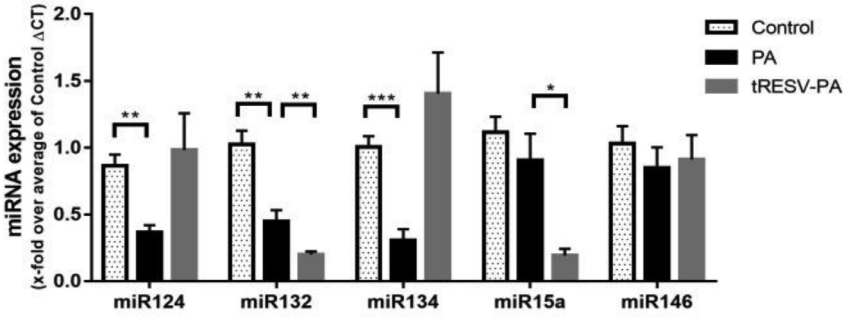

FIGURE 4. *Hippocampal expression (x fold-change) of miR124, miR132, miR134, miR15a and miR146 in control, asphyxia (PA) and tRESV-PA groups relative to control group, using ΔΔCT method. Bars represent mean fold-change ± SEM using $2^{-\Delta\Delta CT}$ formula. * for p = 0.05-0.01, ** for p = 0.01-0.001 and *** for p < 0.001 [Isac, 2017].*

studies [Gao, 2010]. Thus, our results propose a new epigenetic control of neuroinflammation through miR134 [Isac, 2017] (Fig. 4).

Moreover, neuronal maturation might be epigenetically controlled by miR15a [Gao, 2015]. Our data report that tRESV determined a post-asphyxia down-regulation of hippocampal miR15a (Fig. 4), which might accelerate neuronal growth and maturation, with potential resistance to PA.

miR146 is known as an epigenetic regulator of inflammation [Iyer, 2012]. In our study, tRESV did not determined a statistically significant difference in miR146 expression secondary to asphyxia, suggesting that other epigenetic mechanisms such as DNA methylation or histone modification might be involved.

DISCUSSION

Our results show that adding tRESV to maternal diets lowers the post-asphyxia neuroinflammation, as indicated by the lower hippocampal levels of IL-1b and TNFα at 24 and 48 hours, and reduces neural injury, as showed by the lower S-100B hippocampal level, effects that could be associated with the potent antioxidant properties of tRESV and with possible involvement of epigenetic mechanisms. Also, our results point out that long-term addition of low to moderate dose of tRESV to maternal diets might represent an additive factor in increasing hippocampal tolerance to asphyxia.

We also showed that homeostatic synaptic plasticity, axogenesis and neuronal tolerance to asphyxia might involve regulatory epigenetic factors, such as specific miRNA, which could be regarded as promising biomarkers [Isac, 2017]. Ultimately, these findings strongly support the beneficial impact of maternal tRESV supplementation diet on the immature brain of offspring subjected to PA and encourage the study of this topic more extensively in experimental and clinical models.

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